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NF-κB Activation: The IκB Kinase Revealed?

Minireview

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More than a decade ago, the transcriptional activator NF-κB was described as a protein that bound to a specific DNA site in the intronic enhancer of the immunoglobulin κ light chain gene (Sen and Baltimore, 1986). Following the cloning of genes encoding the p50 and p65 subunits of NF-κB, it became evident that both subunits are members of the larger NF-κB/Rel family of transcriptional regulator proteins. Since its initial description, our view of the role of NF-κB in immune and inflammatory responses has broadened significantly (for reviews, see Baldwin, 1996; Baeuerle and Baltimore, 1996).

NF-κB regulation is part of a cellular response system to many different noxious stimuli. NF-κB is activated by a vast number of agents including cytokines like tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), bacterial LPS, viral infection and expression of certain viral proteins like Tax of human T-cell leukemia virus, (HTLV-1), antigen receptor cross-linking of T and B cells, calcium ionophores, phorbol esters, UV radiation, free radicals, endoplasmic reticulum overloading, and others (for reviews, see Verma et al., 1995; Baeuerle and Baltimore, 1996). The genes regulated by the NF-κB family of transcription factors are diverse and include those involved in immune function, inflammatory response, cell adhesion, and growth control (Baldwin, 1996). Recently, the activation of NF-κB has also been linked to the regulation of cell death (Baeuerle and Baltimore, 1996).

NF-κB was initially believed to be lymphoid-specific because of its constitutive presence in the nuclei of mature B cells. In almost all other cells, however, NF-κB is sequestered in the cytoplasm by tightly bound inhibitory proteins called IκBs (Verma et al., 1995). In the family of IκBs, the most important appear to be IκBα, IκBβ, and the newly discovered IκBε. Many of the signals known to activate NF-κB result in phosphorylation and subsequent degradation of the IκBs, allowing NF-κB to translocate into the nucleus and activate target genes. Early studies implicated the phosphorylation of IκB as a central event of this activation and identified potential kinases that were able to phosphorylate the inhibitor protein in vitro (Verma et al., 1995; Baldwin, 1996).

A significant step toward understanding the mechanism of phosphorylation and degradation of IκB was the mapping of the sites phosphorylated in response to NF-κB inducers. Using site-directed mutagenesis, both serine residues S32 and S36 in IκBα were implicated in IκB phosphorylation and degradation in response to TNFα, phorbol 12-myristate 13-acetate (PMA) and ionomycin, as well as a number of other known NF-κB stimuli (Verma et al., 1995; Baldwin, 1996). Interestingly, replacement of S32 and S36 by threonine residues significantly decreases phosphorylation and degradation of the IκBα protein (DiDonato et al., 1996). Another member of the IκB family, IκBβ, is phosphorylated at homologous sites, S19 and S23, upon stimulation with extracellular agents, although with kinetics slower than those of IκBα phosphorylation (DiDonato et al., 1996). There are homologous putative phosphorylation sites on IκBε as well (Baeuerle and Baltimore, 1996).

In vivo, phosphorylation of IκB occurs when it is complexed with NF-κB and does not cause dissociation of the complex. Rather, it signals ubiquitination of IκB, which in turn leads to proteasome-mediated degradation of the inhibitor, releasing free NF-κB (Baldwin, 1996; Baeuerle and Baltimore, 1996).

This understanding of the sites and roles of phosphorylation has allowed a systematic search for the kinase(s) responsible for IκB phosphorylation, a major component of the signal transduction pathways leading to NF-κB activation.

In 1996, Maniatis' laboratory was the first to report the identification of a high molecular weight kinase complex that specifically phosphorylated IκBα at S32 and S36 (Chen et al., 1996). The 700 kDa complex was purified from an unstimulated HeLa cell cytoplasmic extract and required ubiquitin and the ubiquitination enzymes for activity. Although it was shown that a component of the complex is ubiquitinated in vitro and that this event is required for activating the kinase, the target of ubiquitination remains unclear.

Recently, the long efforts of many researchers in the identification of the IκB kinase(s) have been remarkably successful in the molecular cloning and functional analysis of components of an IκB kinase complex (DiDonato et al., 1997; Mercurio et al., 1997; Régnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997).

One approach that led to the molecular identification of two polypeptides of a cytokine-induced IκB kinase complex was the direct biochemical purification of an activity induced by TNFα, which specifically phosphorylates IκBα at S32 and S36 (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). The inducible kinase activity was found in a complex reported to be 500–900 kDa, the IκB kinase or IKK. The IKK complex is composed of several polypeptides, two of which, 85 and 87 kDa in size, copurify with the IκB kinase activity on several columns (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). Through peptide sequencing, the 85 kDa polypeptide was identified as a previously cloned serine-threonine kinase called CHUK (Connelly and Marcu, 1995). The 87 kDa protein was cloned using the same approach (Mercurio et al., 1997; Zandi et al., 1997). Overall, the two polypeptides are 52% identical. They contain an amino-terminal catalytic domain and several putative protein interaction motifs, including a leucine zipper and a helix-loop-helix domain at their carboxyl terminus (Figure 1). In light of its new function, CHUK has been renamed IKKα and the second polypeptide has been designated IKKβ.

A convergent approach, which successfully led to the independent discovery of IKKα, used the known signal transduction pathways downstream of TNF and IL-1 receptors to screen for interacting components. TNF and

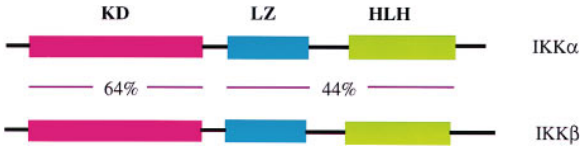

Figure 1. Comparison between IKKα and IKKβ Proteins

(KD) denotes the kinase domain, (LZ) the leucine zipper region, and (HLH) the helix-loop-helix domain. The degree of identity between the kinase domain and the structural motifs of the two proteins is depicted.

IL-1 activate NF-κB via distinct families of cell-surface receptors (Régnier et al., 1997 and references therein; Figure 2). However, both pathways utilize members of the TNF receptor–associated factor (TRAF) family of adaptor proteins as signal transducers. The TRAF proteins share homology at their carboxyl terminus domain, but their binding properties and activities differ. For example, whereas TRAF2 participates in the NF-κB activation by TNF, TRAF6 is involved in NF-κB activation by IL-1 (Figure 2). Recently, it was shown that these different pathways converge at the NF-κB-inducing kinase, NIK (Malinin et al., 1997). NIK has homology to the MAP kinase kinase kinase (MAP3K) family and was first identified by its interaction with TRAF2 (Malinin et al., 1997). NIK activates NF-κB when overexpressed and kinase-inactive mutants of NIK act as dominant-negative inhibitors for both TNF- and IL-1-mediated NF-κB activation. In a yeast two-hybrid screen for NIK-interacting proteins, Régnier et al. (1997) identified IKKα, the same subunit of the kinase complex found by others to phosphorylate IκBα on serines S32 and S36. By searching for IKKα-related kinases, Woronicz et al. (1997) identified IKKβ.

The recent reports assessed the specificity of the IKKα- and IKKβ-associated kinase activities by in vitro phosphorylation assays. Immunoprecipitates of epitope-tagged IKKα and IKKβ, produced either by translation in reticulocyte lysate or expression from transfected plasmids in mammalian cell lines, were employed to phosphorylate IκBα or IκBβ. Based on this assay, it was concluded that an activity associated with IKKα phosphorylates both S32 and S36 of IκBα with the same efficiency (DiDonato et al., 1997; Régnier et al., 1997; Mercurio et al., 1997; Zandi et al., 1997; Woronicz et al., 1997), whereas the phosphorylation of IκBβ seems to be less efficient (DiDonato et al., 1997), and takes place mainly at S23 (Régnier et al., 1997). IKKβ-associated kinase activity seems to be more potent in general (Mercurio et al., 1997; Woronicz et al., 1997) and phosphorylates S19 and S23 of IκBβ equally well (Woronicz et al., 1997). In agreement with previous in vivo data, the IKK complex has a strong preference for serine residues. Replacing S32 and S36 of IκBα protein with threonine residues significantly decreased the efficiency of phosphorylation (DiDonato et al., 1997; Mercurio et al., 1997).

The in vitro results were confirmed by in vivo experiments where the transfection of one of the catalytic subunits of the IKK complex, IKKα, into a cell line with low endogenous IκB kinase activity increased the rate of phosphorylation and degradation of IκBα (DiDonato et al., 1997).

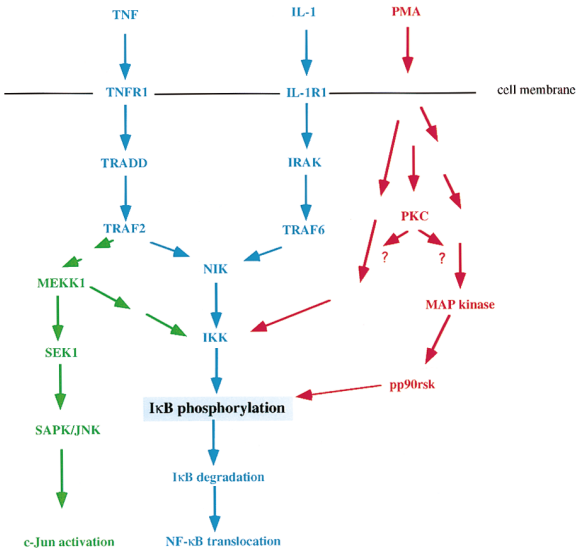

Figure 2. Putative Pathways to NF-κB Activation

See text for details.

Another line of evidence that both IKKα and IKKβ are involved in TNF and IL-1 signaling pathways comes from experiments where anti-sense IKKα (DiDonato et al., 1997) or kinase-inactive IKKα or IKKβ were used to inhibit NF-κB transcriptional activation mediated by TNF and IL-1, or the known downstream components such as TRAF2, TRAF6, or NIK (Régnier et al., 1997; Mercurio et al., 1997; Woronicz et al., 1997). However, it should be pointed out that the extent to which the kinase-inactive mutants inhibit NF-κB activation differs between studies. First, whereas Régnier et al. and Woronicz et al. report that catalytically inactive versions of both IKKα and IKKβ independently block TNF- and IL-1-induced NF-κB-dependent gene activation, Mercurio et al. and Zandi et al. observe little inhibitory effect for the IKKα kinase mutant. This may be due to differences in the mutation that renders the kinase inactive (lysine 44 was mutated to alanine in the former studies, and methionine in the latter). Second, both Mercurio et al. and Zandi et al. report a potent inhibition of nuclear translocation of p65 by the IKKβ mutant, in TNF-treated cells. However, the effect of the IKKα mutant in this assay seems to vary. It is possible that these differences reflect the sensitivity of the assay, or they may indicate a more potent effect of IKKβ. However, it appears that both IKKα and IKKβ are essential contributors to the IKK activity.

The fact that the IKK complex activity is rapidly stimulated by TNF, IL-1, or PMA and the kinetics of activation match those of IκBα phosphorylation and degradation in intact cells (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997) suggests that IKK is a critical kinase complex in vivo. The mechanism by which the complex becomes active in cells is not yet clear. The IKK activity appears sensitive to treatment with the phosphatase PP2A (DiDonato et al., 1997), suggesting that phosphorylation may control its activity. Coexpression of IKKα with NIK seems to lead to the phosphorylation of IKKα and to an increase in its associated kinase activity (Régnier et al., 1997). However, more evidence

Table 1. Mutational Analyses of IKKα and IKKβ

Subunit	Mutation	Effect	Reference
IKKα	In the MAP kinase kinase motif (SxxxS)		
	SS > EE	Minimal enhancement of kinase activity. Low induction of nuclear translocation of p65.	Mercurio et al., 1997
	SS > AA	No inhibition of TNF-induced translocation of p65.	Mercurio et al., 1997
	In the leucine zipper region	Greatly decreased kinase activity. Decreased association with IKKβ.	Zandi et al., 1997
	In the helix-loop-helix region	Greatly decreased kinase activity. Still able to associate with IKKβ.	Zandi et al., 1997
IKKβ	In the MAP kinase kinase motif (SxxxS)		
	SS > EE	Increased kinase activity. Induced nuclear translocation of p65.	Mercurio et al., 1997
	SS > AA	Blocked TNF-induced translocation of p65.	Mercurio et al., 1997
	Deletion of the leucine zipper region	No interaction with IKKα/β, but interaction with NIK unaffected.	Woronicz et al., 1997
	Deletion of the helix-loop-helix region	No effect on interaction with either IKKα/β or NIK.	Woronicz et al., 1997

is needed to conclude whether NIK is directly phosphorylating IKKα.

IKKα and IKKβ can undergo both homotypic and heterotypic interactions as indicated by immunoprecipitation experiments (Mercurio et al., 1997; Zandi et al., 1997; Woronicz et al., 1997), and their oligomerization appears to be mediated by the leucine zipper motif (Woronicz et al., 1997; Zandi et al., 1997; Table 1). Mutational analyses were employed to further assess the role of the motifs present in IKKα/β proteins, as well as a first attempt to understand their regulation. The helix-loop-helix domain seems to be important for the kinase activity (Zandi et al., 1997; Table 1) but does not affect the interaction between the two subunits, or between IKKα/β and NIK (Zandi et al., 1997; Woronicz et al., 1997; Table 1). Both IKKα and IKKβ contain a consensus MAP kinase kinase activation loop motif (SxxxS). Interestingly, mutations of this motif appear to affect mainly the activity of IKKβ (Mercurio et al., 1997; Table 1).

These data suggest that IKKα and IKKβ are crucial for IκB phosphorylation. However, it is still unclear whether these kinases directly phosphorylate IκB. The in vitro studies that apparently show that IKKα or IKKβ can specifically phosphorylate the key serines in IκB have used kinases made either by overexpression in mammalian cells or in vitro translation in reticulocyte lysate. Because of the affinity of the kinases for other components of the IKK complex, especially for NIK, the in vitro analyses may have used IKKα/β complexed to other proteins. It is therefore possible that IKKα/β are not the kinases that directly phosphorylate IκB but rather that they participate in the activation of a kinase that has the true IκB specificity. Identification of the other subunits of the IKK complex and reconstitution of the IκB phosphorylation pathway in vitro from pure components will resolve this issue.

The relationship between the initially described 700 kDa IκB kinase complex of Chen et al. (1996) and the IKK complex characterized by others remains unclear. In particular, Chen et al. found that ubiquitin and ubiquitination enzymes are necessary for kinase activation; however, no requirement for a ubiquitination step in the activation of IKK has been described. It is possible that ubiquitination and phosphorylation may constitute alternative pathways of activation of an IκB kinase complex. Thus, the TNF-induced IKK complex may already be activated, possibly by phosphorylation, making the ubiquitination dispensable. By altering the manner in which the cell extracts are prepared, an IκB kinase complex, similar to the 700 kDa ubiquitination-inducible one, was reported to be activated by TNF treatment of the cells, with the same kinetics as IKK (Lee et al., 1997). The same complex isolated from unstimulated cells could alternatively be activated in vitro by addition of mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), a MAP3K-related kinase of the c-Jun N terminus kinase (JNK) pathway (Lee et al., 1997; Figure 2). In cells, overexpression of MEKK1 led to phosphorylation of IκBα at S32/S36 (Lee et al., 1997). The effect of dominant-negative MEKK1 mutants on the inhibition of NF-κB activation pathway is still ambiguous (Hirano et al., 1996; Lee et al., 1997; Liu et al., 1996). Interestingly, the IKK complex contains MEKK1 as one of its tightly associated subunits, together with another protein involved in the MAP cascade, MAP kinase phosphatase-1 (Mercurio et al., 1997). However, the functional significance of the presence of MAP kinase family proteins in the IKK remains to be established. Thus, only molecular identification of the components of the various complexes will precisely establish the relationship between the kinase activities found to phosphorylate the IκB.

These recent studies elucidate the mechanism of IκB phosphorylation by a cytokine-induced kinase complex, which is able to phosphorylate the IκB at the sites critical for its degradation, causing the subsequent translocation and activation of NF-κB. However, it is still unclear whether all of the diverse stimuli known to activate NF-κB also lead to the activation of IKKα and IKKβ. Two recent reports suggest that the mitogen-activated ribosomal S6 protein kinase, $pp90^{rsk}$, functions as an IκB kinase (Ghoda et al., 1997; Schouten et al., 1997; Figure 2). $pp90^{rsk}$ is activated by phorbol ester, a known inducer of NF-κB, associates in vivo with IκBα, and phosphorylates it mainly at S32 both in vivo and in vitro. Moreover, a dominant-negative form of $pp90^{rsk}$ inhibits IκBα degradation in response to mitogenic stimuli (Schouten et al., 1997). NF-κB activation can also be triggered by double-stranded RNA (dsRNA), and the double-stranded RNA–dependent protein kinase (PKR) can phosphorylate IκB in vitro (Baldwin, 1996). Furthermore, it was shown that in cells lacking PKR, there is a defect in NF-κB activation in response to interferon and dsRNA (Kumar et al., 1997). However, the phosphorylation sites on IκB targeted by PKR and the mechanism of this pathway remain to be established. These results suggest that

IKKα/β may not be unique integrators of the NF-κB response, and that certain stimuli may follow other pathways to IκB phosphorylation and NF-κB activation.

The interesting observation that IKKα and IKKβ differ in their phosphorylation efficiency between IκBα and IκBβ inhibitory proteins, and possibly even between phosphorylation sites on the same molecule, raises the intriguing possibility that the tight regulation of IκB degradation may be achieved by a network of kinases, with different regulation and different preferences for the IκB family members.

The identification of the IKKα and IKKβ kinases is a major step forward in the 10 year effort to understand NF-κB regulation. However, it is by no means the end of the story—at a minimum, identifying the other constituents of the IKK complex will help illuminate the mechanisms involved in control of NF-κB activity. It is likely that a screen for specific IKK inhibitors, led by the pharmaceutical industry, will provide new modulators of inflammatory responses. Such inhibitors could be very valuable as probes for the functions of the individual components of the IKK complex.

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